THE ENZYMES OF HONEY: EXAMINATION BY ION-EXCHANGE CHROMATOGRAPHY, GEL FILTRATION, AND STARCH-GEL ELECTROPHORESIS

JONATHAN W. WHITE, JR. AND IRENE KUSHNIR

Eastern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture, Philadelphia, Pennsylvania 19118, *U.S.A.*

Manuscript received for publication 25th March 1967

SUMMARY

Crude dialysed enzyme concentrates from several bulk honeys, comb honeys, and a 'honey' produced by sugar-feeding of caged bees, have been examined by chromatography on DEAE-cellulose, gel filtration through Sephadex G-200 and starch-gel electrophoresis with a high-resolution enzyme assay procedure.

Ion-exchange chromatography divided crude honey α-glucosidase preparations into 3–9 closely spaced components. A preparation containing no plant constituent, obtained by sugar-feeding caged bees, was relatively unstable. Its α-glucosidase showed only a single very sharp elution peak from DEAE-cellulose; the α-glucosidase elution pattern of a preparation from honey stored by a free-flying colony of bees resembled that from a sample of bulk honey.

All honey α-glucosidase preparations examined showed single bands of activity by Sephadex G-200 gel filtration, with approximate molecular weights about 51 000. Molecular weight of the amylase is about 24 000; for honey glucose oxidase two fractions were obtained, corresponding to molecular weights about 120 000 and > 200 000.

Starch-gel electrophoresis showed the α-glucosidase complex to have from 7 to a maximum of 18 components (isozymes), the latter in a bulk clover honey and the former in a single sample of comb honey. The 13 isozymes of the α-glucosidase of one honey sample had a constant ratio of activity upon sucrose and maltose, further confirming the α-glucosidase nature of honey 'invertase'.

The α-glucosidase complex from sugar-fed 'honey' was much less stable and had a lower migration rate on starch-gel electrophoresis than those from any of the five floral honeys examined, which also varied in apparent rate of migration; this, in conjunction with ion-exchange results, implies an interaction between the bee-introduced α-glucosidase and plant nectar protein components.

INTRODUCTION

The occurrence of enzymes in honey has been known for many years. The voluminous European literature on honey enzymes is largely devoted to methods and results of assays for ' diastase', since this has been used in that continent for at least half a century, to indicate purity and quality of honey. Sucrose-hydrolysing activity in honey is now receiving similar attention. Relatively little consideration has been given to isolation and purification of the various enzymes, or to their characterization. For example, estimates of α-amylase and β-amylase activities and optimal conditions have been made (Lampitt & Bilham, 1936; Kiermeier & Köberlein, 1954) without elimination of the α-glucosidase activity—which could contribute to error by further degradation of maltose and maltotriose, at least. The investigations of Nelson and his colleagues (Nelson & Cohn, 1924; Nelson & Sottery, 1924-25; Papadakis, 1929) on the kinetics of the action of invertase of

honey were carried out with a crude alcohol-precipitated preparation. White and Maher (1953) used a similar preparation to demonstrate the α-glucosidase nature of honey invertase.

As a preliminary to further purification of the common honey enzymes, we have subjected dialysed honey preparations to starch-gel electrophoresis, gel filtration and ion-exchange chromatography on DEAE-cellulose. Assay of the various fractions for amylase, α-glucosidase and glucose oxidase activities, as well as for protein, has provided information useful in separation of these honey enzymes. In addition, evidence has been obtained of interaction between α-glucosidase and nectar proteins and the multiple nature of the α-glucosidase has been demonstrated. Since research on honey at this laboratory has recently been terminated, before purification and characterization of these enzymes could be carried out, the results of these studies are here recorded to avoid future duplication of effort.

EXPERIMENTAL

Materials and methods

Preparation of the dialysed protein and enzyme concentrates from honey has been described (White & Kushnir, 1967), as have the procedures used for starch-gel electrophoresis (White & Kushnir, 1966), gel filtration, and DEAE-cellulose chromatography (White & Kushnir, 1967). The peroxidase-dianisidine assay used for glucose oxidase has been described by Schepartz and Subers (1964), and the α-glucosidase assay procedure by White and Kushnir (1966).

The procedure for amylase in column fractions was adapted from a modification (White, 1959) of the methods of Schade, Marsh and Eckert (1958) and calibrated with honey samples whose amylase values had been determined by that method.

Amylase assay in column fractions

Substrate: 16·7 ml 1% starch prepared as described (White, 1959), 10 ml 0·5 M NaCl, and 6·6 ml 1·6 M phosphate (*p*H 5·3) are made to 100 ml.

Iodine solution: 2·90 ml of iodine stock solution (0·880 g I_2 in 5 ml H_2O containing 2·2 g KCl diluted to 100 ml) and 10 g KI are made to 1 litre.

Procedure: Add 1·0 ml substrate to 0·2 ml sample, incubate 1 hr at 40°. Cool, add 10 ml of iodine solution and determine absorbance at 600 mμ. Calibrate the method with solution of honey of known amylase values, plotting difference in absorbance between average blank and sample against amylase units. An incubation time of 10 min is used for samples which are too active for the 1 hr incubation.

RESULTS AND DISCUSSION

Ion-exchange chromatography

Enzyme preparations (0·25 – ·50 ml) were absorbed on a column (0·8 × 18 cm) of DEAE-cellulose previously equilibrated with 0·01 M potassium phosphate (*p*H 8·0) and eluted with this buffer. After about 25 2·0 ml fractions, a linear salt

gradient reaching 0·5 M at fraction 100 was applied. Fig. 1*a* shows the elution of α-glucosidase when a sodium chloride gradient is applied; Fig. 1*b* is typical of

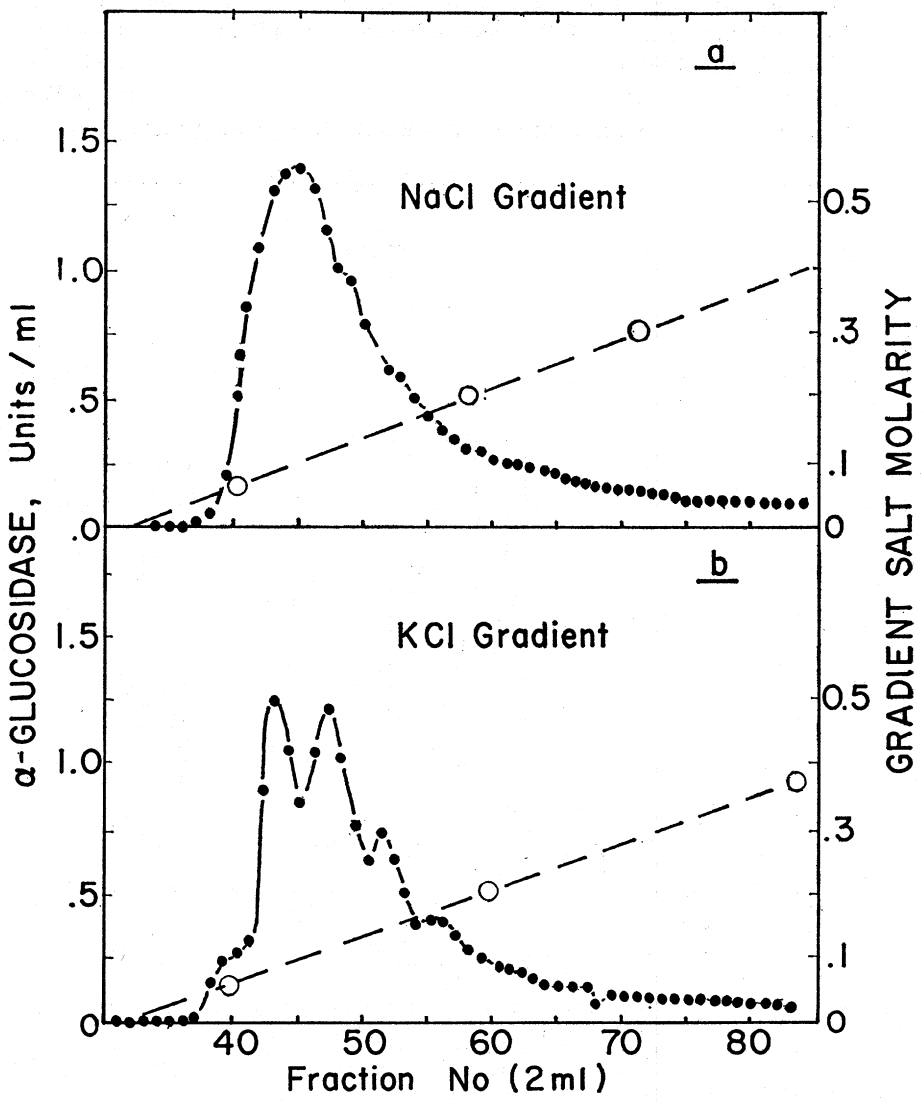


FIG. 1. Chromatography on DEAE-cellulose (0·8 × 18 cm bed) of α-glucosidase preparations from cotton honey (HS 38)
Column equilibrated with 0·01 M potassium phosphate buffer (*p*H 8·0), and eluted with same buffer with gradient of NaCl (1*a*) or KCl (1*b*) applied as shown by broken line (scale right) (note that the abscissa scale differs from that elsewhere.)

the elution curve when a potassium chloride gradient is applied. The improvement in resolution is obvious. All subsequent elutions (except those with sodium borate columns) were done with KCl gradients.

Fig. 2 shows a typical elution of 'protein' (measured by the Layne (1957) calculation of the Warburg and Christian optical method) and the α-glucosidase under these conditions. The amount of protein not held by the column varied considerably among samples, depending upon age of preparation and its concentration. In this example 27% of the material passed rapidly through the column, the remaining 73% (14·9 mg) being eluted by the KCl gradient as shown by the broken line. On the other hand, of the 271 units of α-glucosidase applied to the column, only 27% was retained on the column, 73% passing through rapidly.

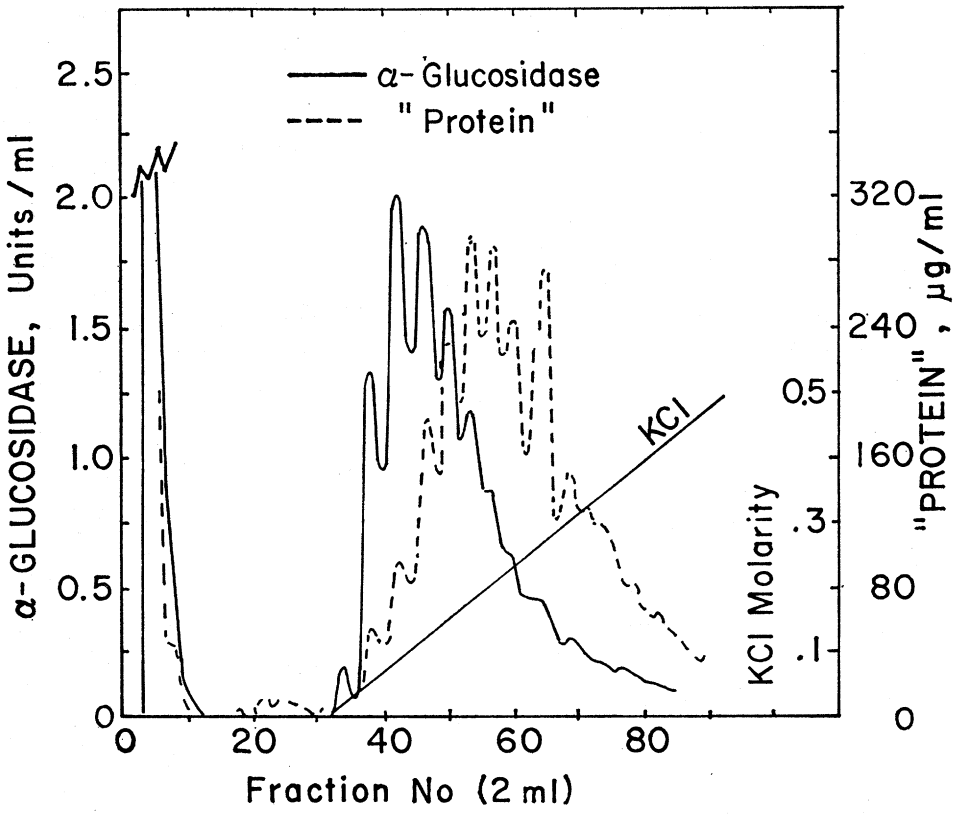


Fig. 2. Chromatography on DEAE-cellulose of a α-glucosidase preparation from cotton honey (HS 38), elution with 0·01 M potassium phosphate (*p*H 8·0); KCl gradient as shown Solid line: α-glucosidase activity (scale left); broken line: 'protein' measured by optical method (scale right). 'Protein' retained and fractionated, 14·9 mg (73%); α-glucosidase retained and fractionated, 73·5 units (27%).

The non-retention of the enzyme and protein material is not caused by exceeding the exchange capacity of the column. This is shown by reapplication of material not retained to another column. In Fig. 3*a* is shown ion-exchange chromatography of an α-glucosidase preparation from aster-goldenrod honey (HS 37). Here 47% passed rapidly through the column, and 53% (68 units) was retained and eluted by application of the gradient. The appropriate tubes were combined, dialysed, concentrated, and reapplied to fresh DEAE-cellulose columns of the same size.

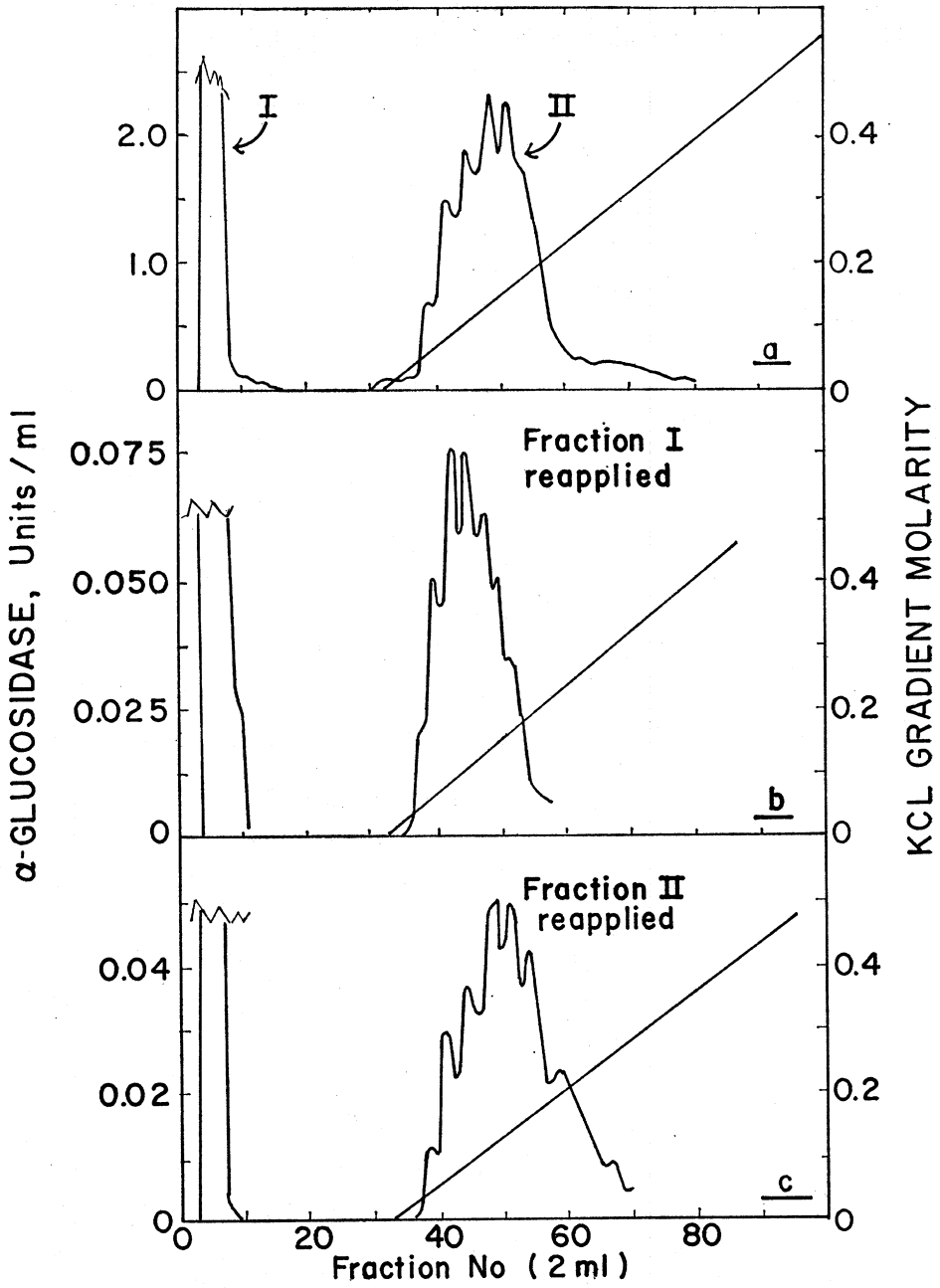


Fig. 3. Chromatography on DEAE-cellulose of α-glucosidase as in Fig. 2

3*a*: Preparation from goldenrod-aster honey (HS 37). Adsorbed, 68 units (53%).

3*b*: Pooled fractions I (3 – 10) from 3*a* reapplied to fresh column. Adsorbed, 1·65 units (11%).

3*c*: Pooled fractions II (35 – 60) from 3*a* applied to fresh column. Adsorbed, 1·56 units (17%).

Results are shown in Fig. 3*b* and 3*c*. Most of the enzyme activity passed rapidly through both columns, although the total applied was much less than

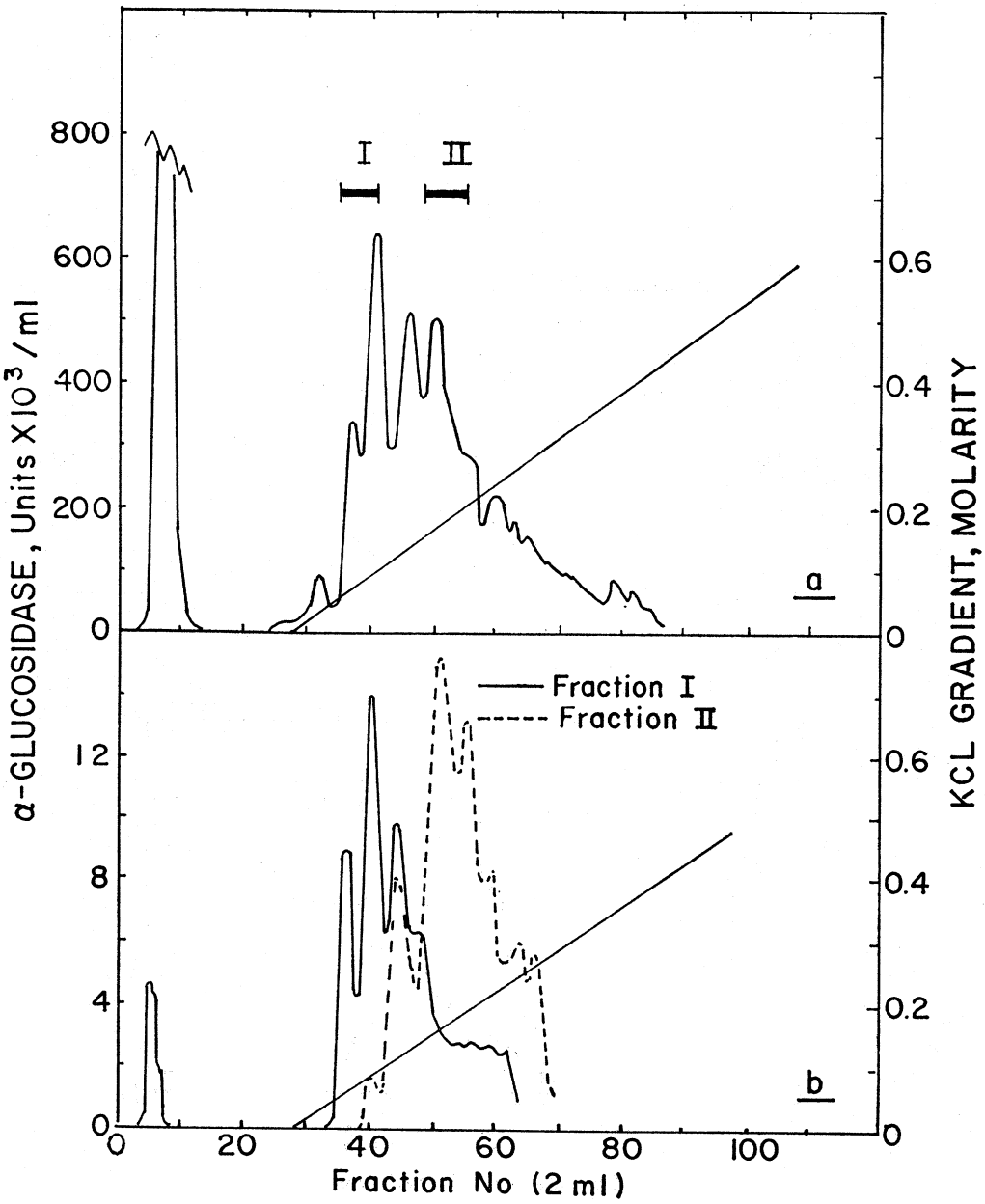


Fig. 4. DEAE-cellulose chromatography

4*a*: α-glucosidase preparation from cotton honey (HS 38)

4*b*: Rechromatography of pooled fractions I (35 – 40) and II (48 – 55) on fresh columns, after dialysis and concentration

that retained on the first passage through the exchanger. Of the enzyme passing through the first column without retention (Fraction I), 86% again did so (Fig. 3*b*). Of the enzyme retained in its first passage through the column (Fraction II), 83% passed through immediately on the second passage; thus no difference between the two fractions I and II in Fig 3*a* was found. Physical change could have taken place during the treatment between passages through the column (dialysis and concentration by Aquacide I).

In another experiment the adsorbed and unadsorbed fractions from DEAE-cellulose columns were filtered through Sephadex G-200 columns. No difference in retardation of the α-glucosidase was found, implying that the two fractions from the ion-exchange column do not differ appreciably in molecular weight. Difference may be due to degree of accessibility of binding groups in two forms of the enzyme.

Examination of the elution diagrams in Fig. 2 – 5 shows that the material eluted by the KCl gradient consists of several closely spaced components, varying in number and relative intensity. Evidence that this fractionation is real was obtained by combining the fractions from the DEAE-cellulose separation shown in Fig. 4*a* into two portions and reapplying each to a fresh column after dialysis, concentration by Aquacide I and redialysis to remove material introduced from the Aquacide.

The elution diagrams for the two α-glucosidase fractions after reapplication to the columns are seen in Fig. 4*b*. It is evident that these fractions do indeed differ in terms of their relative content of the several sub-fractions. This is also apparent in Table 1, which gives the KCl concentration at which each enzyme peak was

TABLE 1. Reapplication of pooled DEAE-cellulose column fractions to another column; KCl molarity at maxima of enzyme activity

	Whole preparation * (Fig. 4*a*)		*Fraction I* (Fig. 4*b*)		*Fraction II* (Fig. 4*b*)	
	KCl at peak	*Relative activity*	*KCl at peak*	*Relative activity*	*KCl at peak*	*Relative activity*
	0·03 M	12		0		0
I	0·065	53	0·06 M	64		0
I	0·095	100	0·09	100	0·09 M	10
	0·13	81	0·12	70	0·12	53
II	0·17	81		0	0·17	100
II	0·20	44		0	0·20	86
	0·23	34		0	0·22	53
	0·26	28		0	0·27	39
	0·27	23		0	0·28	37

* Fractions comprising peaks enclosed within boxes were pooled, dialysed, concentrated, redialysed and applied to separate columns with results shown.

eluted, and also the relative enzyme activities of the sub-bands for each column separation.

The peak at 0·095 M KCl was strongest in Fraction I as selected, and is also the principal peak after reapplication to the column. The enzyme band at 0·17 M KCl predominated in Fraction II as selected, and also dominates the elution pattern

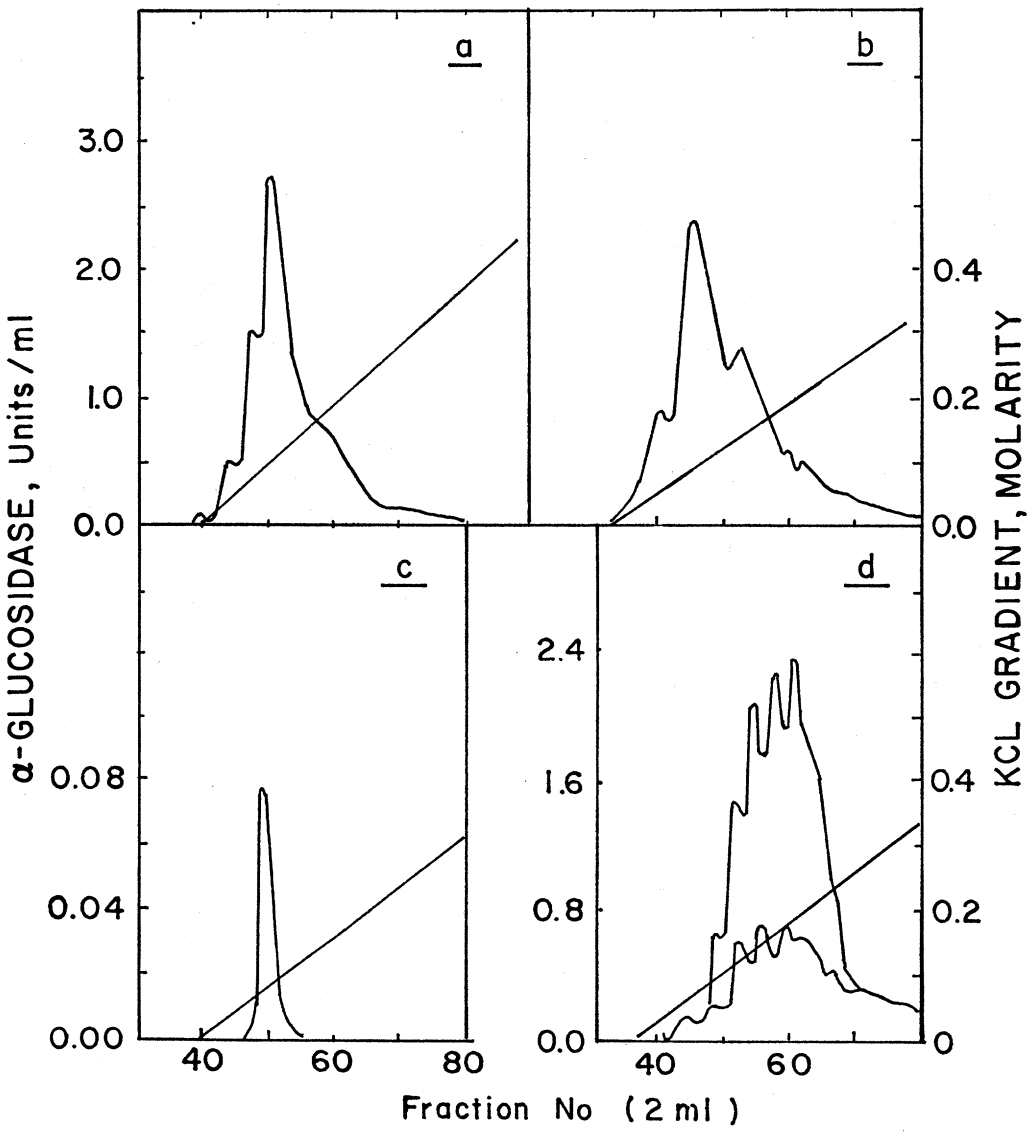


Fig. 5. Chromatography on DEAE-cellulose of various α-glucosidase preparations

5a: Bulk clover honey (HS 36)

5b: single-comb *Lespedeza* (359)

5c: stores from sugar-fed confined bees (M259)

5d: upper curve, unheated goldenrod-aster (HS 37); lower curve, same honey heated with 53% activity remaining

after reapplication of the fraction to the second column. It is concluded that the band structure obtained by DEAE-cellulose chromatography of honey α-glucosidase under the conditions used here is real. In general, 5 – 9 such maxima may be distinguished in the α-glucosidase elution curves, excluding the non-adsorbed portion. The salt concentration at which each maximum occurs is reproducible to about ± 0·01 M.

When preparations from different honey samples are subjected to ion-exchange chromatography, the α-glucosidase elution curves show general resemblance with reproducible differences in their finer structure. Fig. 5 shows this for three honey types and (5*c*) for a preparation made from food stores produced by caged bees fed sucrose (White & Kushnir, 1967). In the last, α-glucosidase activity was quite low, and also much less than that from natural honey. The overall α-glucosidase recovery from the column is ordinarily 60 – 80%; with the sugar-fed honey it was only 3·7%. The preparation from clover honey (low in α-glucosidase and protein, 5*a*) was also less stable than that from honeys with higher protein content and higher activity, and showed greater losses during preparation.

Of the honey types whose α-glucosidase elution curves appear in Fig. 1 – 5, most represent the product of an indeterminate number of colonies of honeybees, being from bulked extracted honey, and representing an entire apiary or a blend from several apiaries. It is conceivable that the finer structure in the α-glucosidase —and protein (White & Kushnir, 1967)—zones from the ion-exchange columns might originate from minor genetic or strain differences among bees of different colonies or races. The product whose α-glucosidase elution is seen in Fig. 5*c* was stored by a small colony of bees from a single queen. To ascertain if the simplicity of this α-glucosidase is due to this factor, an enzyme concentrate was prepared from a sample of comb honey (hence stored by a single colony) and subjected to chromatography on DEAE-cellulose. Fig. 5*b* shows the result. The α-glucosidase elution curve of this sample (a *Lespedeza* honey) is quite similar to that of Fig. 5*a*, which is from a bulked clover honey. Complexity of the 'protein' by ultraviolet analysis and by the Lowry procedure was also seen to be similar for the two honey preparations (White & Kushnir, 1967).

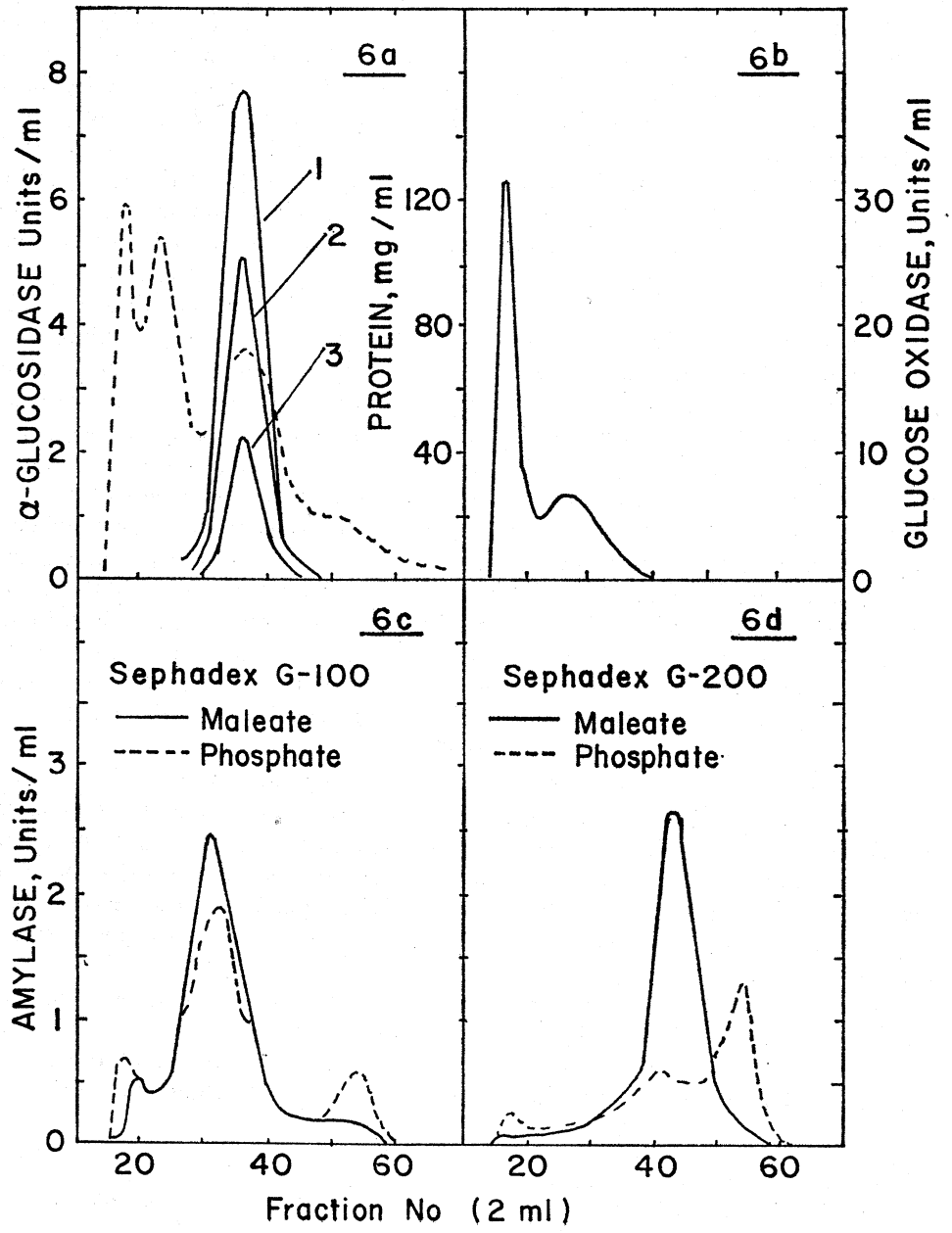
A sample of goldenrod-aster honey (HS 37) was heated for 5 hr at 60°. Its α-glucosidase activity decreased 53%, from 95·7 to 50·6 u/g, in reasonable agreement with a half-life of 4·7 hr at this temperature calculated from other samples (White, Kushnir & Subers, 1964). DEAE-cellulose chromatography of a preparation from such honey showed no striking qualitative differences from unheated honey in its α-glucosidase elution curve, though the total recovery of activity was lower.

Fig. 5*d* shows the α-glucosidase elution curves from the unheated honey (upper) and the same honey after heating (lower); both curves represent the same amount of original honey. The lower curve is less than half of the height of the upper curve because only 30% of the applied activity was recovered from column.

Gel filtration

When enzyme preparations were subjected to gel filtration on Sephadex G-200

in 0·01 M phosphate (*p*H 6·5), all the α-glucosidase activity was found in a single band. Irrespective of origin of the honey preparation, all appeared at the same position relative to the void volume, within experimental error; the same was true when a sample of whole honey was passed through the column. Approximation of the molecular weights by the equation of Leach and O'Shea (1965) (who used



G-200) gave values between 48 000 and 55 000 for α-glucosidase preparations from four honey types.

Fig. 6*a* shows the α-glucosidase activity of filtrates from Sephadex G-200 columns of preparations from three samples—two honeys and stores from sugar-feeding of bees. For comparison, the protein content of the fractions is also shown for one preparation from honey.

When amylase activity was determined on Sephadex columns eluates, a more complex pattern resulted. Amylase activity was shown in the most retarded portion of the elution curves from Sephadex G-25, G-100 and G-200, the relative amount increasing in this order. This is shown in Fig. 6*c* and 6*d* (broken lines) for G-100 and G-200. To determine if this retardation is an interaction of the amylase with the carbohydrate (modified dextran) material in the presence of phosphate, the same preparation was filtered through columns of G-100 and G-200, prepared with 0·01 M maleate buffer (*p*H 6·5) instead of phosphate, and washed with maleate. The considerable improvement in elution pattern resulting is shown by the solid lines in Fig. 6*c* and 6*d*. Interaction with the column resulting in excessive retardation is greatly reduced, the resulting bands being far more symmetrical. Applying the calculation described by Whitaker (1963) (who used G-100), the approximate *apparent* molecular weight for the principal amylase peak in phosphate on G-100 (Fig. 6*c*) is 23 200 and that of the most retarded peak 1 350. The value calculated for the single maximum in maleate on G-100 is 24 600. When the *apparent* molecular weights of peaks obtained from the G-200 gel filtration in phosphate are similarly calculated (Leach & O'Shea, 1965), the vestigial peak at about fraction 40 (Fig. 6*d*) yields 21 000, and the principal peak 4 460. The single peak in maleate on G-200 corresponds to a value of 21 600. These values are only approximations, since the filtrations were not carried out for this purpose. Selby and Maitland (1965) reported a cellulase fraction of apparent molecular weight 5300 in phosphate buffer, which was retarded to the same extent on reapplication to G-75; since it was excluded from G-25, they accepted the apparent value as indicative of a small molecule. No other buffer system was used.

Our material was also excluded (in phosphate) from G-25; with G-50 the peak followed the exclusion volume by only 2 ml, but in both cases a very small portion was retarded to the end of the elution. This retarded portion was propor-

Fig. 6. Gel filtration on Sephadex columns (bed volume 2·1 × 31 cm) of enzyme preparations from honey

6*a*: G-200 in 0·01 M phosphate (*p*H 6·5): solid lines (scale left)

(1) α-glucosidase from 0·5 ml preparation equivalent to 1·11 g goldenrod-aster honey (HS 35)

(2) α-glucosidase from 0·5 ml preparation equivalent to 5·52 g clover honey (HS 36)

(3) α-glucosidase from 0·25 ml preparation equivalent to 8·9 g of stores from sugar-fed bees (M259).

Broken line (scale right), protein (Lowry) from clover honey (HS 36) as above

6*b*: G-200 in 0·01 M phosphate (*p*H 6·5): glucose oxidase from 0·5 ml preparation equivalent to 4·19 g cotton honey (HS 38) (scale right)

6*c*: amylase from cotton honey (HS 38) on G-100

Broken line, 0·5 ml equivalent to 4·1 g honey, in 0·01 M phosphate (*p*H 6·5).

Solid line, 0·5 ml equivalent to 5·7 g honey, in 0·01 M maleate (*p*H 6·5)

6*d*: Same preparations as 6*c* on G-200

tionally larger in G-100, and in G-200 (Fig. 6*d*) it represented a major part of the activity. Since buffer change restored the elution curve to a reasonable value, no reapplication of retarded fractions to columns was done.

Fig. 6*d* shows the filtration of glucose oxidase activity in 0·01 M phosphate (*p*H 6·5) through Sephadex G-200. Substitution of maleate had no effect on the results. About half the activity was excluded from the G-200 column, implying a molecular weight well in excess of 200 000; the retardation of the remaining half corresponds to a molecular weight of approximately 120 000.

Starch-gel electrophoresis

Preparations were subjected to starch-gel electrophoresis as detailed in another publication (White & Kushnir, 1966). The gels were sliced lengthwise into slabs, one of which was stained for proteins by nigrosine and another cut into 1-mm slices and assayed for α-glucosidase activity. Results of the protein staining appear elsewhere (White & Kushnir, 1967). In a few instances where only location of the enzyme region was required, 2- or 5-mm slices were analysed.

As we have previously noted (White & Kushnir, 1966), use of 1-mm slices reveals considerably more detail in the electrophoretic diagram than is apparent when the activity is shown by directly staining the gel or using 2-mm slices. From 8 to about 20 closely spaced bands of α-glucosidase activity, depending on the sample, are partially resolved by this procedure. They occur in groups of 2 or 3 and are fairly reproducible in both number and relative activity. Under the conditions employed (16 hr in borate, *p*H 8·9 3·5 v/cm) the active area covers about 35 – 45 mm of gel.

To determine if these sub-bands are real (Cann, 1966), a migration was carried out using a relatively concentrated preparation, giving the pattern seen in Fig. 7*a*. The lack of detail in the pattern is caused by the high concentration of enzyme (White & Kushnir 1966). From a parallel sample, four 1-mm slices were removed, reinserted into the origin of a second starch gel and again subjected to electrophoretic migration. Fig. 7*b*, 7*c*, 7*d* and 7*e* show the patterns obtained from each slice. It is clear that the four fractions differed materially and show a logical relation to each other. Thus the sub-bands are electrophoretically distinct.

Identity of honey 'invertase' and 'maltase'

The invertase of honey was shown (White & Maher, 1953) to be a gluco-invertase, transferring glucose to suitable acceptors. Appreciable maltase activity and action on melezitose were noted; thus the enzyme appeared to be an α-glucosidase.

Efforts to separate the activities against sucrose and maltose by several means (partial inactivation by heat, oxidizing or reducing agents, fractionation on alumina, paper electrophoresis) were unsuccessful. For further information on this point, an enzyme preparation from an autumn flower honey was subjected to starch-gel electrophoresis.

The gel was cut into 1-mm slices in the active area. The extracts of these slices were divided and allowed to act upon sucrose and maltose solutions (5%,

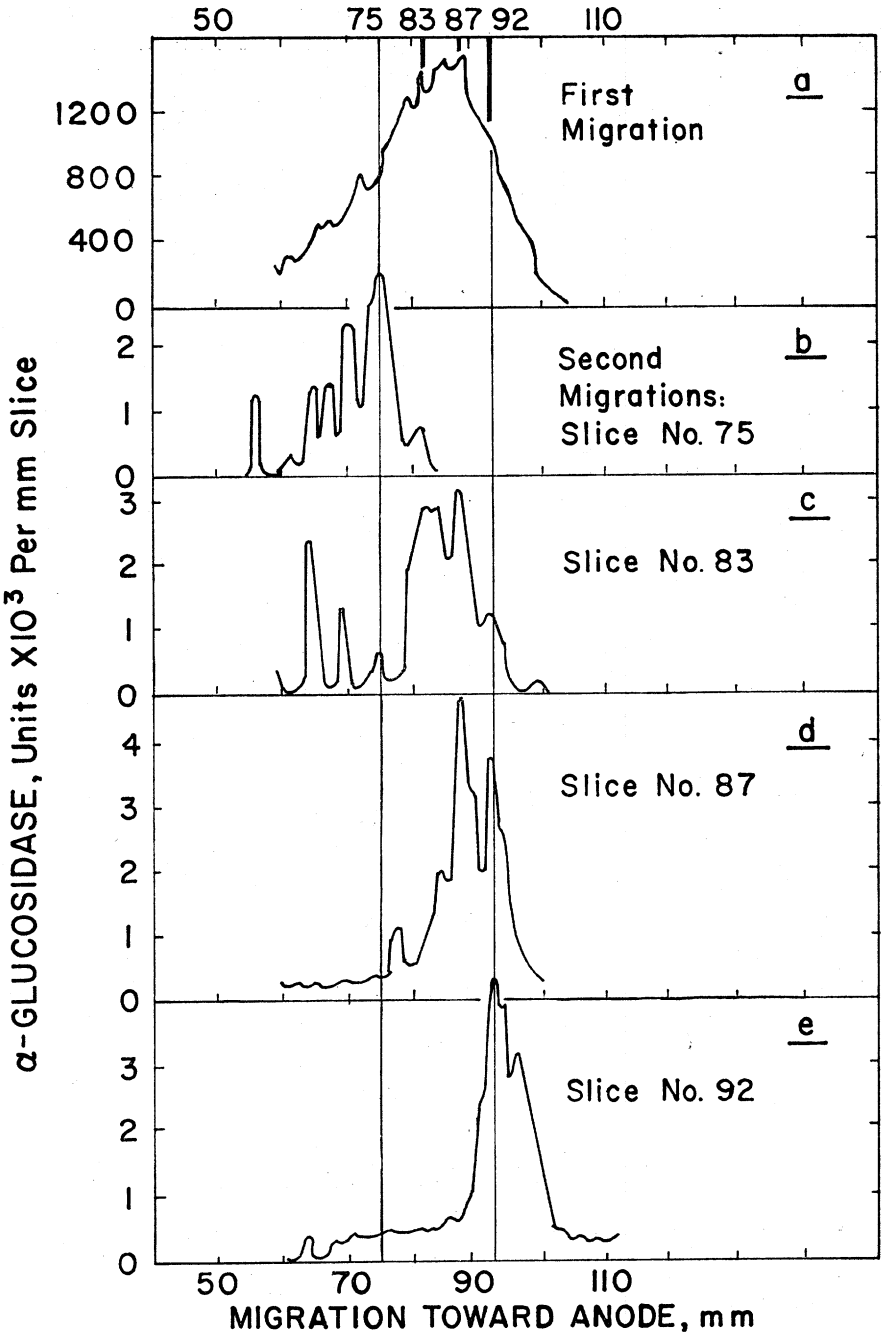


Fig. 7. Starch-gel electrophoresis of α-glucosidase preparation from a goldenrod-aster honey, borate (*p*H 8·9), 3·60 v/cm, 16 hr.
7*a*: Whole preparation, from which four 1-mm slices (numbered at top) were taken for subsequent migrations
7*b*–7*e*: Indicated single slice inserted at origin of fresh gel and re-run under the same conditions; all on same gel

0·05 M acetate *p*H 5·9). The maltose was previously purified of contaminant glucose by large-scale charcoal column fractionation to attain sensitivity to low glucose concentrations by avoiding excessive background colour when assaying for glucose. Fig. 8 shows the electrophoretic pattern of the 'invertase' and 'maltase' so

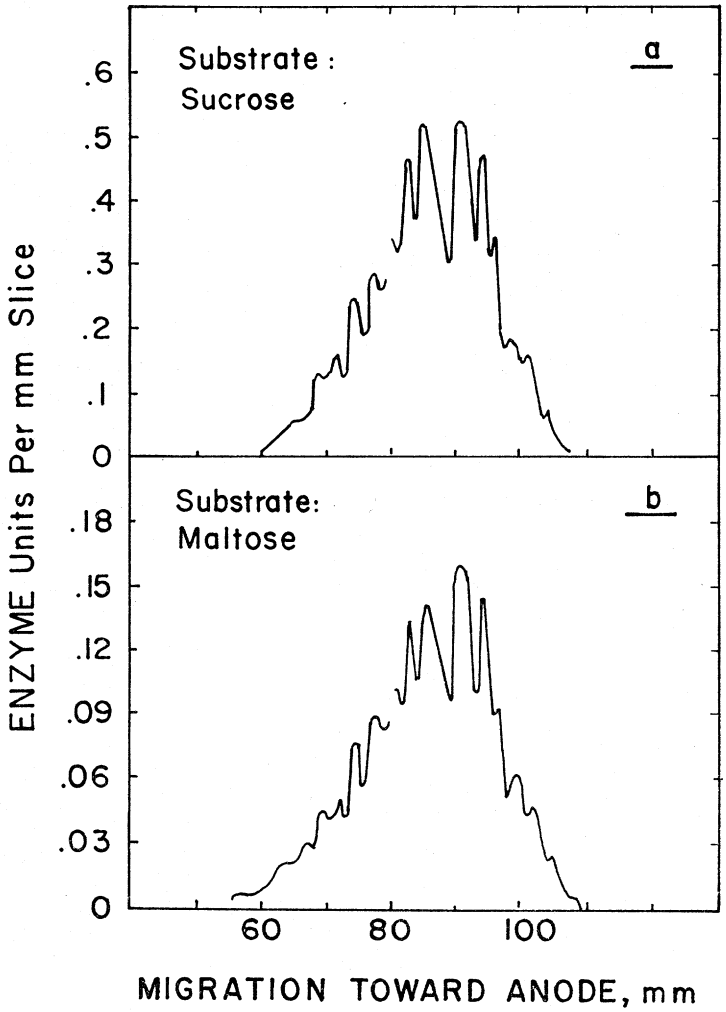


Fig. 8. Starch-gel electrophoresis of enzyme preparation from a goldenrod-aster honey (HS 37), 4·0 v/cm, borate (*p*H 8·9), 16 hr. Slices (1-mm) extracted (see text) and activity of extract determined on sucrose (8*a*) and maltose (8*b*)

measured. The two patterns are identical in shape; activity upon sucrose is consistently 3·30 times that upon maltose over the entire electropherogram, involving 50 pairs of determinations. It is reasonable to conclude that these two activities reside in a single enzyme.

Effect of honey heating on α-glucosidase pattern

Processing of honey to delay granulation and effect pasteurization can be accomplished by heat without damage to honey quality (White, Kushnir & Subers, 1964). Among tests for excessive heating of honey used in some countries are assays for amylase and sucrase activities. All the honey samples used in this study were unheated. It is of interest to examine the effect of honey heating on the electrophoretic pattern of the α-glucosidase. Fig. 9 shows the electrophoretic

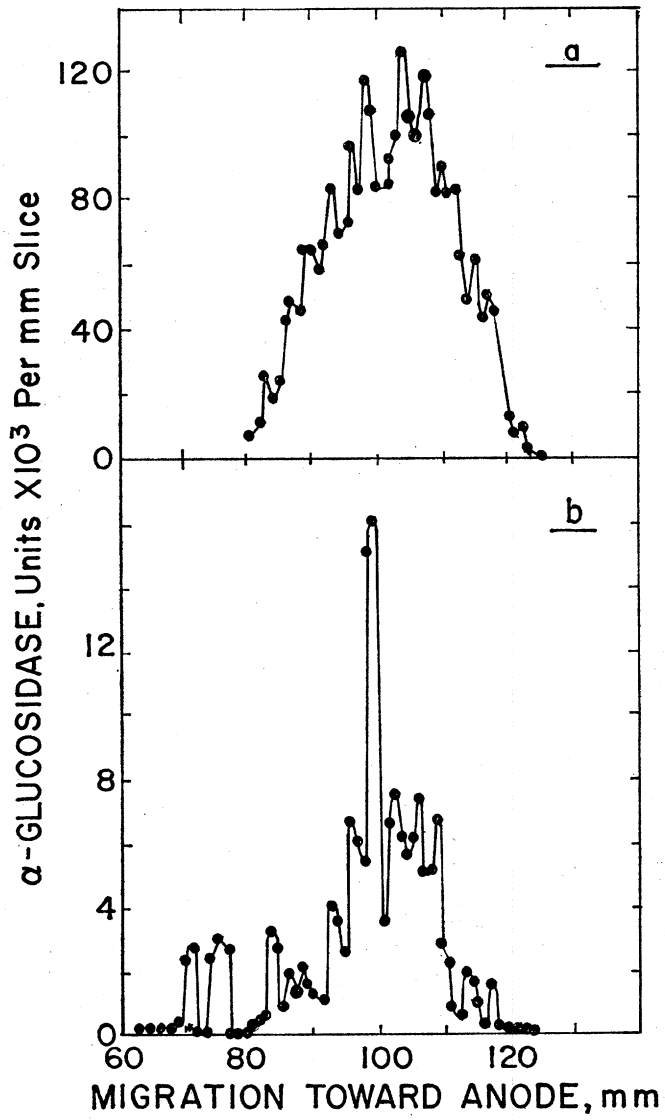


Fig. 9. Starch-gel electrophoresis of enzyme preparation from a goldenrod-aster honey (HS 37)
9*a*: Preparation from unheated honey
9*b*: Preparation from honey heated 5 hr. at 60°

patterns of an enzyme preparation from the original honey (9*a*) and from the heated honey (9*b*), described above. Differential sensitivity to heat of the various isozymes is apparent, with some bands considerably more heat-stable than the others; such differentiation was not evident when the separation was by DEAE-cellulose chromatography. Each of the relatively more stable bands in Fig. 9*b* is fixed by at least two experimental points.

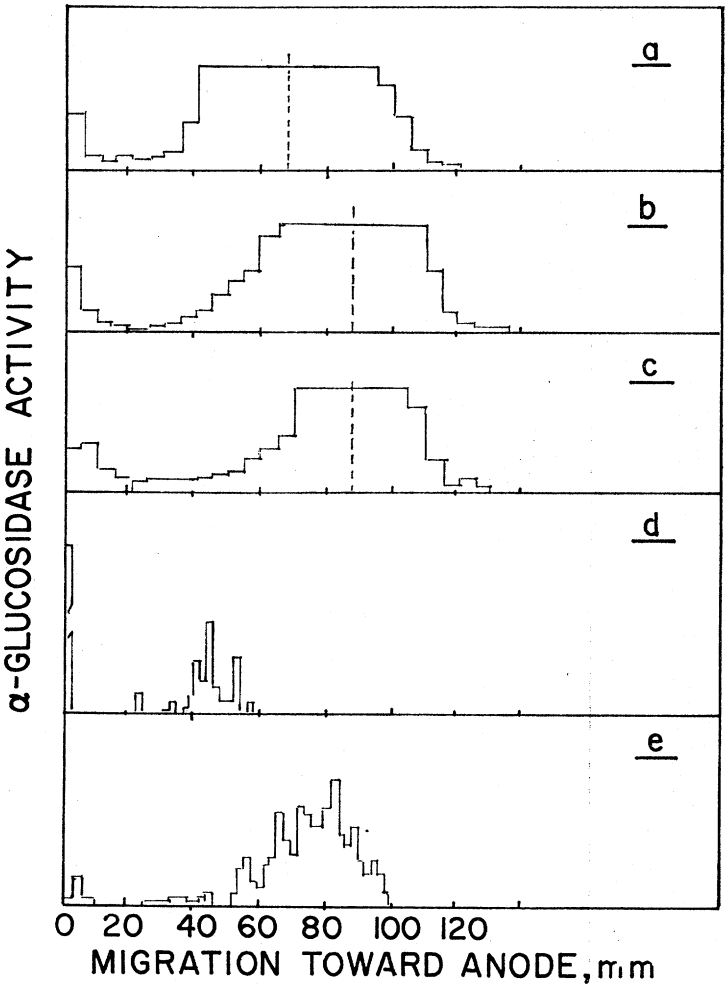


Fig. 10. Preliminary migration of α-glucosidase preparations on starch-gel, borate (*p*H 8·9), 3·70 v/cm, 17 hr.

10*a*: from clover honey (HS 36), 290 units/ml

10*b*: from goldenrod-aster honey (HS 37), 740 units/ml

10*c*: from cotton honey (HS 38), 400 units/ml

10*d*: from stores of sugar-fed bees (M259), 20 units/ml

10*e*: from clover honey (HS 36), 19 units/ml

a, b, c on same gel, 5-mm slices; *d, e* on same gel, 2-mm slices

Since the source of α-glucosidase in honey is the honeybee, it was thought that different bulk honey samples* would not show significant differences in the electrophoretic pattern of their enzyme. Preliminary migrations are shown in Fig. 10. Three bulk honeys were tested on the same gel, taking 0·5-cm slices to locate the enzyme area. No effort was made to adjust concentrations to reagent capacities, hence Fig. 10*a*, 10*b* and 10*c* show locations only, and not precise structure or relative intensities. In Fig. 10*a*, a clover honey, the midpoint of the active area is at about 68 mm, whereas for 10*b* (goldenrod-aster) and for 10*c* (cotton) it is at about 87 mm. The preparation from cotton honey is spread less widely than that from goldenrod-aster; the most widely spread is the first (clover). Differences in enzyme concentration applied to the gel are not large enough to cause these effects.

The two bottom diagrams in Fig. 10 are also from the same gel. Fig. 10*d* is the pattern (2-mm slices) from a 'honey' obtained by feeding confined bees with sugar (White & Kushnir, 1967). It is seen to migrate much more slowly than that in Fig. 10*e* which is from 2-mm slices of the clover honey of Fig. 10*a*. This sugar-fed sample (which contains no plant protein or other plant constituents) consistently showed a more slowly migrating and less complex pattern than those of the bulk floral samples examined.

The electrophoretic α-glucosidase patterns for two of the bulk samples were determined with more resolution. In Fig. 11 the electrophoretic distribution in starch gel of the α-glucosidase from the clover honey and the goldenrod-aster honey are shown with 1-mm resolution. Both samples were run on the same gel; normally variation in position of bands in replicate patterns across the 12-cm gel width is of the order of 1 – 2 mm. The migration rates of the α-glucosidase complex of the two preparations are seen to differ significantly, as does the number of components present. The preparations were adjusted to equivalent activities before migration.

The two samples were from bulked honey, originating in an unknown number of hives, apiaries, producers, or even geographical areas. Thus the multiplicity of sub-bands of α-glucosidase might represent a combination of relatively simple patterns from various strains or even races of bees. Some light is thrown on this by the examination of the enzyme pattern of honey from a single comb taken from a colony in which all the worker bees had the same mother (except those that may have joined the colony by drifting), though not necessarily the same male parent.

Preparations of α-glucosidase were made from two samples of comb honey as described above. Fig. 12*a* and 12*b* show the electrophoretic patterns obtained from these single-colony honeys, both on the same gel. The patterns indeed appear less complex than those in Fig. 11. This does not result from the greater dilution (*ca* 40 units/ml) of the solutions applied for Fig. 12*a* and 12*b* than for those in Fig. 11 (*ca* 100 u/ml); a pattern shown by the sample in Fig. 11*a* is spread as widely when diluted to 20u (Fig. 10*e*), though when the concentration is much higher (*ca* 300 u/ml) the spread is about 65 mm (Fig. 10*a*) instead of 50 mm. With

*By bulk honey is meant extracted honey from many colonies, blended by the producer or packer.

only seven components, the pattern in Fig. 12*a* is the most simple yet encountered under high-resolution conditions. But the somewhat greater complexity of Fig. 12*b* (9 components), which is also from a single colony of bees, and run on the same gel, does not allow us to ascribe the complexity of the bulk-honey patterns (Fig. 11) entirely to the presence of enzymes from a large number of colonies of bees.

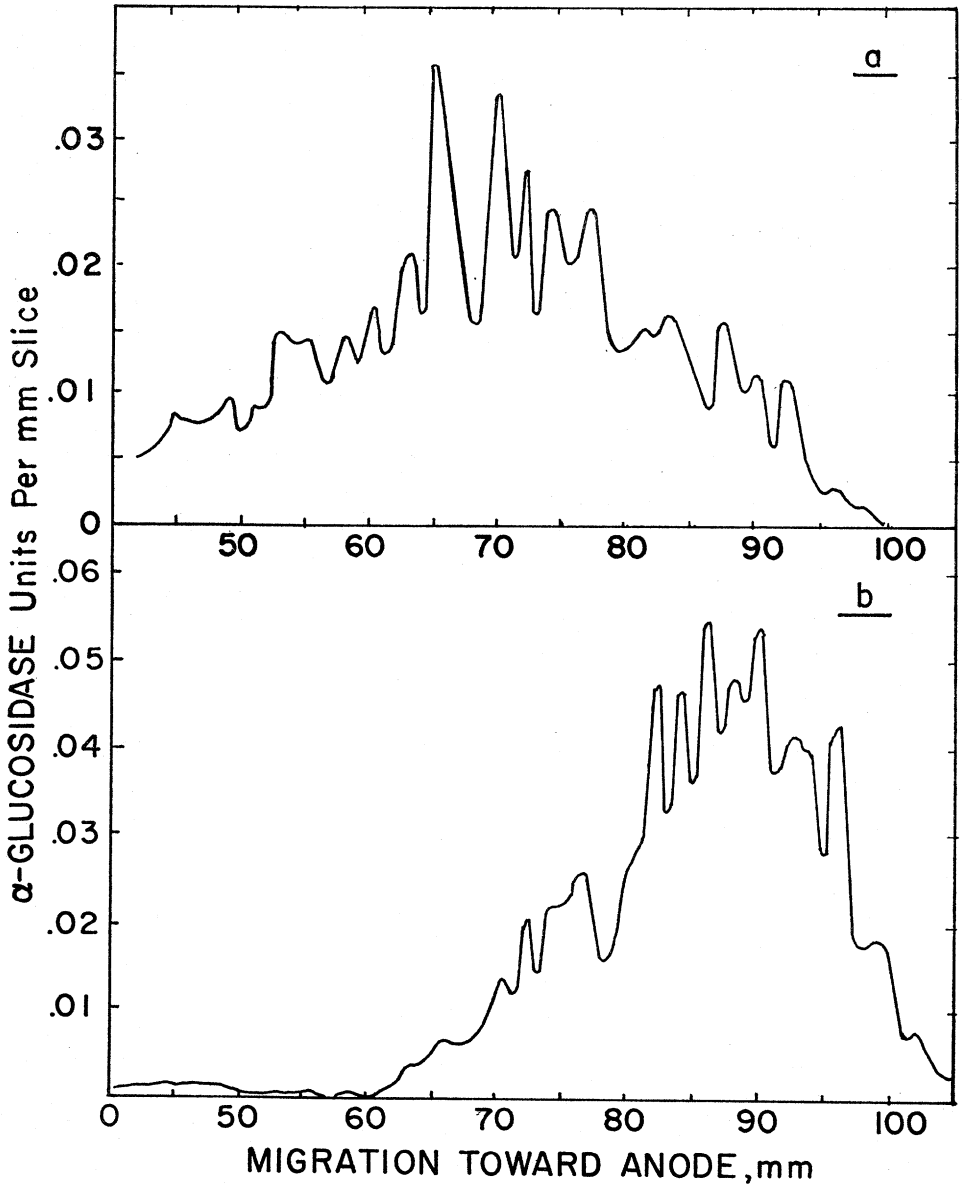


Fig. 11. Starch-gel electrophoresis of α-glucosidase preparations from honey, both on same gel, borate (*p*H 8·9), 3·70 v/cm, 16 hr. (note expanded abscissa)

11*a*: Clover honey (HS 36), 96 units/ml

11*b*: Goldenrod-aster honey (HS 37), 105 units/ml

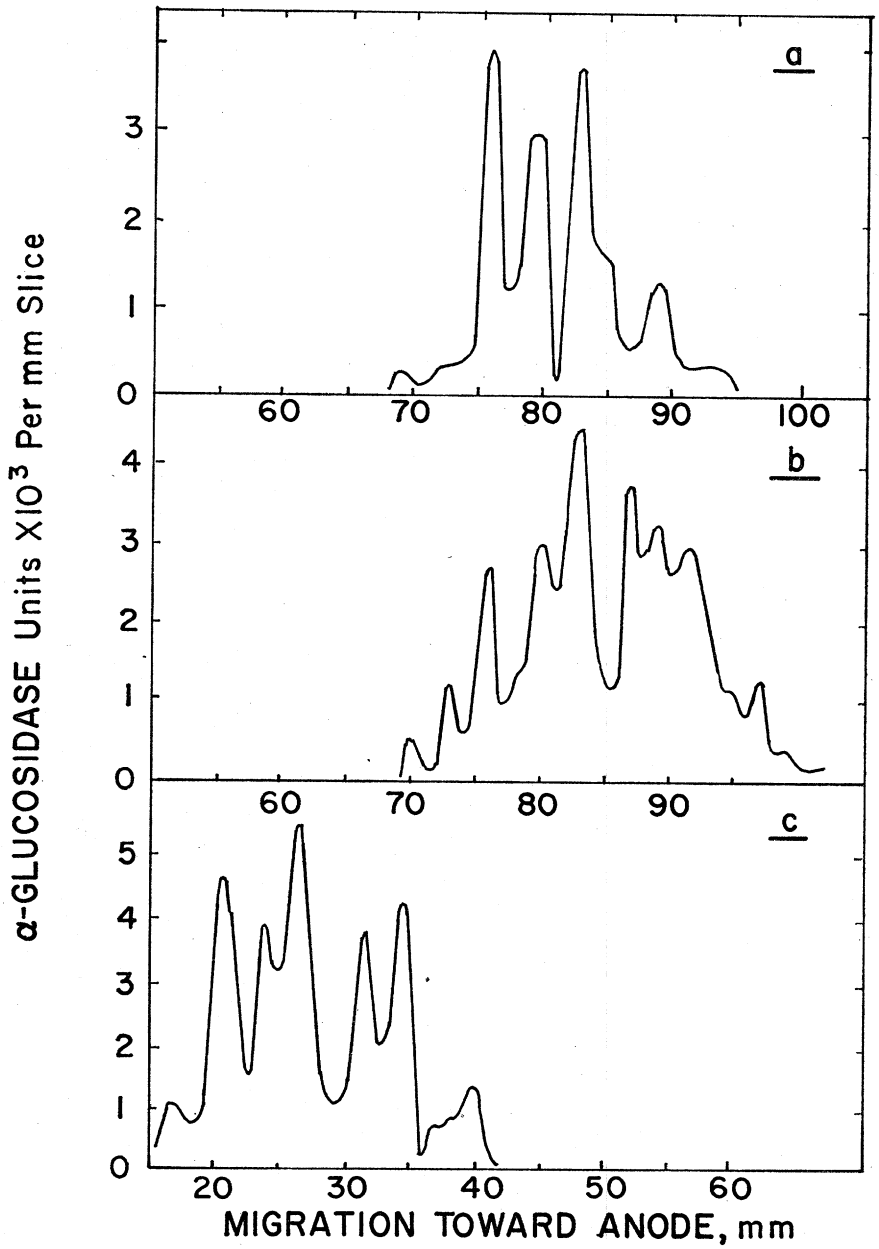


Fig. 12. Starch-gel electrophoresis of α-glucosidase preparations from single-comb (i.e. single colony) honey samples, all at 3·52 v/cm, borate (*p*H 8·9), 16 hr, 12*a* and 12*b* on same gel; note different abscissa scales.

12*a*: *Lespedeza* honey, 38 units/ml

12*b*: Mixture of wingstem (*Actinomeris alternifolia*) and *Lespedeza*, 30 units/ml

12*c*: Stores from sugar-feeding of bees, 44 units/ml.

All these α-glucosidase patterns are of enzyme from honey of free-flying bees working natural nectar sources. Fig. 12*c* shows the α-glucosidase pattern from the sample of 'honey' produced by caged bees from a single colony fed sucrose solution, without access to natural nectar sources. Such possible sources of plant material as nectar contained in the bees when removed from the colony were eliminated by a starvation period followed by a sugar-feeding and storing period. The combs thus used were then replaced by water-washed combs for collection of the sample (White & Kushnir, 1967).

The pattern, with 7 – 8 components, is intermediate in complexity between the other two in Fig. 12; further, it migrates at less than half the rate (this is also seen in Figs. 10*d* and 10*e*). Repetition of these observations with additional samples should be carried out for definitive results, but it appears to imply in floral honey a plant nectar contribution to the enzyme complex which increases electrophoretic mobility. It is unlikely that this is due to the retarding effect of gel structure on mobility, since in gel filtration experiments the α-glucosidase from all samples (including this one) appear at the same intermediate V/V_0 on columns of Sephadex G-200, and hence must have approximately equivalent molecular weight.

CONCLUSIONS

In the work reported, efforts were made to demonstrate the real nature of the fractionations observed, by resubmission of fractions and zones to the separating treatment. In all cases it can be concluded that the properties of the fractions obtained during elution from ion-exchange columns actually differed. Similarly, re-insertion of zones from gel electrophoresis into subsequent gels yielded differing patterns related to the differing migration of the originals.

The α-glucosidase of honey is shown by ion-exchange chromatography and by high-resolution starch gel electrophoresis to consist of a varying number of isozymes. Gel filtration shows that all fall within a narrow range of molecular weight. An equilibration in solution among structures of similar molecular weight adsorbed and not adsorbed by DEAE-cellulose has been demonstrated. Evidence is given favouring a stabilizing interaction of the honeybee α-glucosidase with plant nectar components, probably proteins. Possibly the low enzyme levels among some types of honey can be ascribed in part to the low protein content of some of these; additional evidence is needed on this.

It is not clear why the numbers of isozymes varied to such an extent among the samples. The lowest numbers (7) were from single-comb (single-colony) samples, whereas 18 were found in a bulk honey sample. Whether the variation is caused by genetic differences among bees, or varietal differences among plants, cannot be stated at this time. An obvious extension of this work is the examination of the α-glucosidase isozymes from single bees and the effects of genetic variability. The gel electrophoretic and assay methods described here are sufficiently sensitive to permit this application. There is no doubt that the invertase activity and the maltase (α-glucosidase) activity of the honey reside in the same enzyme, since constant ratios of the two activities were found for all 13 isozymes of the sample examined.

Approximate molecular weight for the α-glucosidase preparations was about 51 000. Honey amylase in maleate buffer showed an approximate molecular weight of 24 000, but in phosphate an apparent interaction with Sephadex resulted in anomalous values. Two active fractions were obtained by gel filtration of honey glucose oxidase, with approximate molecular weights of 120 000 and > 200 000.

ACKOWLEDGEMENT

We greatly appreciate the co-operation of Dr. N. E. Gary, University of California, Davis, who prepared the sample of stores from sugar-fed bees at Cornell University.

REFERENCES

CANN, J. R. (1966) Multiple electrophoretic zones arising from protein-buffer interaction. *Biochemistry, N.Y.* 5 : 1108 – 1112

KIERMEIER, F. & KÖBERLEIN, W. (1954) Ueber die Hitzeinaktivierung von Enzymen in Honig. *Z. Lebensmittelunters. u. -Forsch.* 98 : 329 – 347

LAMPITT, L. H. & BILHAM, P. (1936) Notes on the absorption spectra of honey. *Chemy Ind.* 14 : 71 – 72

LAYNE, E. (1957) Spectrophotometric and turbidimetric methods for measuring proteins. Pages 447 – 454 from *Methods in enzymology* ed. S. P. Colowick and N. O. Kaplan, Vol. 3 *New York : Academic Press*

LEACH, A. A. & O'SHEA, P. C. (1965) The determination of protein molecular weights of up to 225 000 by gel-filtration on a single column of Sephadex G-200 at 25° and 40°. *J. Chromat.* 17 : 245 – 251

NELSON, J. M. & COHN, D. J. (1924) Invertase in honey. *J. biol. Chem.* 61 : 193 – 224

NELSON, J. M. & SOTTERY, C. T. (1924) Influence of glucose and fructose on the rate of hydrolysis of sucrose by invertase from honey. *J. biol. Chem.* 62 : 139 – 147

PAPADAKIS, P. E. (1929) Further findings on invertase from honey. *J. biol. Chem.* 83 : 561 – 568

SCHADE, J. E., MARSH, G. L. & ECKERT, J. E. (1958) Diastase activity and hydroxy-methyl-furfural in honey and their usefulness in detecting heat alteration. *Fd. Research* 23 : 446 – 463

SCHEPARTZ, A. I. & SUBERS, M. H. (1964) The glucose oxidase of honey. I. Purification and some general properties of the enzyme. *Biochim. biophys. Acta* 85 : 228 – 237

SELBY, K. & MAITLAND, C. C. (1965) The fractionation of *Myrothecium verrucaria* cellulase by gel filtration. *Biochem. J.* 94 : 578 – 583

WHITAKER, J. R. (1963) Determination of molecular weights of proteins by gel filtration on Sephadex. *Analyt. Chem.* 35 : 1950 – 1953

WHITE, J. W., JR. (1959) Report on the analysis of honey. *J. Ass. off. agric. Chem.* 42 : 341 – 348

WHITE, J. W., JR. & KUSHNIR, I. (1966) Enzyme resolution in starch gel electrophoresis. *Analyt. Biochem.* 16 : 302 – 313

——— (1967) Composition of Honey. VII. Proteins. *J. apic. Res.* 6(3) : *In press*

WHITE, J. W. JR., KUSHNIR, I. & SUBERS, M. H. (1964) Effect of storage and processing temperatures on honey quality. *Fd. Technol. Champaign* 18 : 555 – 558

WHITE, J. W., JR. & MAHER, J. (1953) Transglucosidation by honey invertase. *Archs. Biochem. Biophys.* 42 : 360 – 367